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Different Patterns of Regulation for the Copper and Cadmium Metallothioneins of the Ectomycorrhizal Fungus *Hebeloma cylindrosporum*▽

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Metallothioneins (MTs) are small cysteine-rich peptides involved in metal homeostasis and detoxification. We have characterized two MT genes, Hc*MT1* and Hc*MT2*, from the ectomycorrhizal fungus *Hebeloma cylindrosporum* in this study. Expression of Hc*MT1* and Hc*MT2* in *H. cylindrosporum* under metal stress conditions was studied by competitive reverse transcription-PCR analysis. The full-length cDNAs were used to perform functional complementation in mutant strains of *Saccharomyces cerevisiae*. As revealed by heterologous complementation assays in yeast, Hc*MT1* and Hc*MT2* each encode a functional polypeptide capable of conferring increased tolerance against Cd and Cu, respectively. The expression levels of Hc*MT1* were observed to be at their maximum at 24 h, and they increased as a function of Cu concentration. Hc*MT2* was also induced by Cu, but the expression levels were lower than those for Hc*MT1*. The mRNA accumulation of Hc*MT1* was not influenced by Cd, whereas Cd induced the transcription of Hc*MT2*. Zn, Pb, and Ni did not affect the transcription of Hc*MT1* or of Hc*MT2*. Southern blot analysis revealed that both of these genes are present as a single copy in *H. cylindrosporum*. While the promoters of both Hc*MT1* and Hc*MT2* contained the standard stress response elements implicated in the metal response, the numbers and varieties of potential regulatory elements were different in these promoters. These results show that ectomycorrhizal fungi encode different MTs and that each of them has a particular pattern of expression, suggesting that they play critical specific roles in improving the survival and growth of ectomycorrhizal trees in ecosystems contaminated by heavy metals.

Ectomycorrhizal fungi enhance the growth and fitness of their host plants, mostly by improving their mineral nutrition (45). They also increase the tolerance of the host plants to heavy metals (5, 12, 15, 19) and improve the survival and growth of trees in ecosystems contaminated by heavy metals, such as Cu, Cd, Pb, Ni, or Zn (21). This capability is of key ecological importance, since heavy metals are continuously being dispersed into the biosphere by natural processes or human activity and can constitute a serious environmental hazard (3).

Metal resistance is a result of different mechanisms, such as restriction of metal uptake, increased efflux, and extracellular or intracellular complexation. Jacob et al. (21) showed that complexation of Cd by metallothioneins (MTs) is a key mechanism for Cd tolerance in the ectomycorrhizal fungus *Paxillus involutus*. MTs are ubiquitously present in eukaryotic and prokaryotic organisms (see reference 10 and the references therein). They are characterized by their small size (<7 kDa), high content of the amino acid Cys (up to 33%), and high degeneracy rate for the remaining residues; they are encoded by a multigene family and contain metal binding Cys-rich domains (10). MTs are involved in metal homeostasis and detoxification (10, 16). Transcription of MTs is typically induced by the same metal ion(s) that binds to the protein, thus providing a direct activation of their protective function (49).

Seven members of the MT gene family have been identified in *Arabidopsis* spp. (10); each of them shows a unique pattern of expression and regulation. Likewise, mammalian MTs are encoded by a large family of different MT genes that vary in their responses to different inducers (24). Among fungi, *Candida glabrata*, like higher eukaryotes, contains a large family of MT genes. However, the transcription of *C. glabrata* MT genes is activated in response to Cu and Ag but not by other heavy metals, such as Cd, a potent activator of higher-eukaryote MTs (32). In contrast, only two MTs have been identified for *Saccharomyces cerevisiae*. The first one is encoded by the *CUP1* locus and is induced by Cu and Ag but not by other heavy metals (8, 40). The second one, *Crs5*, is regulated by Cu, Zn, and oxidative stress (14, 41). An MT has been identified for *Agaricus bisporus* (37), *Gigaspora margarita* (29), *Neurospora crassa* (38), *Pyrenopeziza brassicae* (44), *Podospora anserina* (2), and *P. involutus* (6). Noticeably, a Cu binding MT was purified from the ectomycorrhizal fungus *Laccaria bicolor* (19). Further investigations are needed to identify different members of the MT family in ectomycorrhizal fungi and to study their regulation of expression in an attempt to determine their respective functional roles in heavy metal detoxification and/or tolerance.

The aim of the present work was to identify MTs of the ectomycorrhizal fungus *Hebeloma cylindrosporum* and to document the specificities of their induction. Our results show that this fungus encodes at least two different MTs, which are spe-

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cifically induced by Cu and Cd, respectively, but not by Zn, Pb, and Ni or oxidative stress. The functionality of these two *H. cylindrosporum* MTs was verified through complementation of the growth deficiency of *S. cerevisiae* mutants.

MATERIALS AND METHODS

Organisms and growth parameters. The monokaryotic strain h1 of the ectomycorrhizal fungus *H. cylindrosporum* was obtained from single-spore germination (50). It was maintained on agar medium, as described by Rouillon et al. (43), that contained modified Melin's medium (33) supplemented with Heller's micronutrients (23). The yeast strains used for heterologous expression of Hc*MT1* and Hc*MT2* were two copper-sensitive strains, DTY3 (*MAT*α *leu2-3 112his3*Δ*1 trp1-1 ura3-50 gal1 CUP1*^{s}) and DTY4 (the same with *cup1*::*URA3*), referred to as *cup1*^{s} and *cup1*$^\Delta$ (31), and one cadmium-sensitive *yap1* strain (*MAT*α *his3D1 leu2D0 met15D0 ura3D0 YML*::*kanMX4 yap*::*RP1*) of *S. cerevisiae* derived from the wild-type strain BY4741 (*MAT*α *his3 leu2 met15 ura3*).

Metal tolerance and growth conditions. The response of *H. cylindrosporum* to various concentrations of metals was assessed by growing the pure mycelial culture in liquid medium (pH 5.6 ± 0.2). The fungus was grown in different concentrations of Cu (0, 80, 160, 240, and 320 μM as $CuSO_4 \cdot 5H_2O$), Cd (0, 7, 14, 21, and 28 μM as $3CdSO_4 \cdot 8H_2O$), Zn (0, 1, 2, 4, 6, 8, and 10 mM as $ZnSO_4 \cdot 7H_2O$), Pb (0, 25, 50, 75, and 100 μM as $PbCl_2$), and Ni (0, 85, 170, 250, and 350 μM as $NiSO_4 \cdot 6H_2O$). The mycelium was harvested after 21 days of incubation at 25°C and washed with distilled water, and the dry biomass was measured. To study the MT induction by different heavy metals, the fungus was first grown in Melin's liquid medium for 2 weeks. The mycelium was then transferred to fresh medium containing different concentrations of heavy metals, as mentioned above. To study the optimum time for maximum induction of MT, the fungus was grown in Melin's medium with a 320 μM concentration of Cu and a 21 μM concentration of Cd for different time intervals (0, 12, 24, 36, 48, 60, and 72 h). Oxidative stress treatment was imposed by incubating the culture supplemented with 25 mM H_2O_2 and harvesting the mycelium at the different time intervals mentioned above.

DNA and RNA isolation. Genomic DNA was isolated from the mycelium of *H. cylindrosporum* as described by van Kan et al. (48). The total RNA was extracted from the mycelium by using Trizol reagent (Invitrogen Life Technologies). The concentration of total RNA was determined by measuring the UV absorbance at 260 nm. The RNA purity was checked by determining the A_{260}/A_{280} ratio, and its integrity was checked by formaldehyde agarose gel electrophoresis.

cDNA synthesis. cDNA was synthesized using approximately 5 μg of total RNA with a RevertAid first-strand cDNA synthesis kit (Fermentas Life Sciences). Oligo$(dT)_{18}$ primer was added, and the final volume was adjusted to 20 μl with diethyl pyrocarbonate-treated water. This mixture was denatured at 70°C for 5 min and put on ice to allow the primers to anneal to the template. Samples were reverse transcribed for 60 min at 42°C with Moloney murine leukemia virus reverse transcriptase (RT) enzyme (Fermentas Life Sciences), RNase inhibitor, and deoxynucleoside triphosphates (dNTPs). The reaction was stopped by incubation for 10 min at 70°C.

PCR amplification. The PCR was performed with primers HcMT1F (5′-AAGCTTGCGGTTCTGACAAT-3′) and HcMT1R (5′-GGCTGGACAAAATCGAACTC-3′) to amplify Hc*MT1* and primers HcMT2F (5′-CTCTTCCTGCACCTGCCACTT-3′) and HcMT2R (5′-ACGAGGAAACTTGGCAGAAG-3′) for Hc*MT2*. PCR was carried out in a final volume of 50 μl containing 1× reaction buffer, 2.5 U *Taq* polymerase (Invitrogen Life Technologies), primers (0.2 μM), $MgCl_2$ (1.5 mM), dNTPs (100 μM), template DNA (100 ng), and nuclease-free water. The PCR program was as follows: initial denaturation at 95°C for 5 min; 30 cycles of 1 min at 92°C, 1 min at 55°C, and 1 min at 72°C; and a final extension at 72°C for 5 min. PCR products were separated on 1.2% (wt/vol) agarose gels and visualized by ethidium bromide staining.

Cloning and sequencing. The PCR products amplified from genomic and cDNA were extracted and purified from agarose gels using QIAquick columns (Qiagen, Valencia, CA) and directly cloned into a pGEM-T Easy vector (Promega). DH5α *Escherichia coli* competent cells were transformed and plated onto Luria agar with ampicillin medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG). The transformants were randomly selected, and plasmid DNA was prepared with a Qiagen plasmid mini kit. DNA sequences were determined using T7 and SP6 primers and an ABI310 DNA sequencer (ABI, Foster City, CA). Sequence analysis was performed with the BLAST program (1) by using the nucleic acid and predicted amino acid sequences deposited in multiple databases.

RACE-PCR. To find the complete sequence of Hc*MT1* and Hc*MT2*, rapid amplification of cDNA ends PCR (RACE-PCR) was used to isolate the 5′ and 3′ ends of cDNA according to the protocol provided by the manufacturer (5′/3′ RACE kit; Roche). For 5′ RACE, 5 μg of total RNA was reverse transcribed at 55°C for 60 min in a 20-μl reaction, by using HcMT1R primer (gene specific), and first-strand cDNA was further purified. Homopolymeric [oligo(dA)]-tailed cDNA was synthesized according to the manufacturer's instructions. Five microliters of oligo(dA)-tailed cDNA was then amplified in the presence of 1× buffer, 1 mM $MgCl_2$, 2 mM of each dNTP, 1.6 μM of primer HcMR (5′-GACCGGGATGAATTGAGAGA-3′), and 1.6 mM oligo(dT) anchor primers for the first PCR. The first PCR product was gel purified and further amplified by anchor primer and the HcMR primer, and the amplified products were cloned and sequenced as mentioned earlier. For 3′ RACE, 5 μg of total RNA was transcribed by using oligo(dT) anchor primer, and this first-strand cDNA was amplified by HcMT1F and anchor primer. Amplified 3′ RACE products were cloned and sequenced as mentioned earlier. In the case of the Hc*MT2* gene, primer HcMT2R was used for 5′ RACE and primer HcMT2F was used for 3′RACE.

Cloning of Hc*MT1* and Hc*MT2* genes and promoter regions. The genomic clones of Hc*MT1* and Hc*MT2* and the adjacent DNA sequence, ~1.5 kb upstream from the Hc*MT1* and Hc*MT2* start codon of *H. cylindrosporum*, were cloned using a Clontech Universal Genome Walker kit (Clontech Laboratories, Inc.). Briefly, *H. cylindrosporum* genomic DNA was digested with one of three different blunt-end restriction enzymes (EcoRV, PvuII, or DraI). An Hc*MT1*-specific primer (5′-GTGGTAATGCGTACGAGCTGTTGCAGTTGCAG-3′) and an Hc*MT2*-specific primer (5′-GTCAAGGGAAGAAACGTTTCTCAAAAGCTGACCCAG-3′) were designed and used in combination with the adapter-specific primer AP1 to amplify large genomic segments adjacent to these genes. A nested PCR was performed with other primers for Hc*MT1* (5′-TGGCAAGAGCCAGAGGAGCAGGTGCAACT-3′) and Hc*MT2* (5′-GTCCGAGTCGAACGATTTGTCGGCCCTCACGAC-3′), which were designed from upstream sequences of the previous primers by using adapter-specific nested primer AP2. PCR products were gel purified with a Qiagen QIAquick gel extraction kit and directly cloned into a pGEM-T Easy vector (Promega). DH5α *E. coli* competent cells were transformed and plated onto Luria agar with ampicillin medium containing X-Gal and IPTG. The transformants were randomly selected, and plasmid DNA was prepared with a Qiagen plasmid mini kit. DNA sequences were determined using T7 and SP6 primers and internal primers using an ABI310 DNA sequencer (ABI, Foster City, CA). The upstream sequences (~1,200 bp) of Hc*MT1* and Hc*MT2* were further analyzed using Genomatix software (9) and TRANSFAC databases for potential transcription factor binding sites or elements (http://helixweb.nih.gov/transfac/). Both Genomatix and TransFac software programs provide a high level of confidence in the prediction of regulatory elements based on multiple matches and conservation of key nucleotide sequences (51). For example, Genomatix scores of 0.9 or above indicate a very high confidence level for the predicted regulatory elements. We have listed the elements that scored 0.9 or above and confirmed these scores with high-scoring values on Transfac.

Transcript quantification using competitive reverse transcription-PCR. Total RNA was isolated from the metal-treated mycelium by the Trizol method. Reverse transcription (RT) was performed with total RNA as mentioned above. mRNA accumulation of Hc*MT1* and Hc*MT2* was quantified by competitive RT-PCR (cPCR). The cPCR is based upon the coamplification with the same primer pair of the target sequence (to be quantified) with a known amount of DNA fragment (competitor) which differs in size from that of the target. After reverse transcription of all mRNAs from different treatments using poly(dT) primers, an internal fragment of the Hc*MT1* and Hc*MT2* cDNAs and a competitor DNA were coamplified by PCR using the same set of primers specific for the Hc*MT1* and Hc*MT2* sequences mentioned above. The competitor sequence was a plasmid-cloned genomic DNA fragment, which was 490 bp in length for Hc*MT1* and 295 bp for Hc*MT2*. In a PCR containing a cDNA sample and a known amount of plasmid-cloned competitor, primers HcMT1F and HcMT1R amplify simultaneously the 295-bp-long cDNA fragment and the 490-bp-long competitor fragment. In the case of Hc*MT2*, 210-bp-long cDNA fragments and the 295-bp-long competitor fragments were obtained with HcMT2F and HcMT2R primers. Owing to these size differences, amplified sequences were easily separated by electrophoresis in 1.5% agarose gels, and their relative amounts were quantified using a gel documentation system (UltraLum) after ethidium bromide staining of the gels.

PCRs were performed with a final volume of 25 μl using 1.0 μl of RT-cDNA, 0.2 μM primers, 1.5 mM $MgCl_2$, 100 μM dNTPs, 1.25 U *Taq* polymerase, and 1× reaction buffer (Invitrogen Life Technologies) with an appropriate amount of competitor. Amplification conditions consisted of 3 min at 94°C, 25 cycles of 1

min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 5 min at 72°C. Preliminary experiments showed that at 25 cycles, the PCR was still in the exponential phase. Standard curves were constructed by coamplifying different known amounts of target DNA with a constant amount of competitor DNA. A standard curve was obtained by plotting the log values of the amplification ratios of target DNA/competitor DNA against the log values of the amplified target DNA (picograms of target DNA) added to the PCR mix before amplification. RNA was extracted from three independent batches of each treatment. Two reverse transcription reactions were performed from each extract, and three to four cPCRs were carried out for each cDNA sample. Each experiment was repeated three times. The transcript level was expressed using arbitrary units, where 1 corresponds to the expression level either at zero time or for the control.

Yeast functional complementation assays. The full-length Hc*MT1* and Hc*MT2* sequences were amplified using MT1F (5′-CGGGATCCATGCAATTCACTTC TATCCTCGTC-3′) and MT1R (5′-CCGGAATTCTCAGTTGCAGTTGCAG TTGTTGG-3′) and MT2F (5′-CGGGATCCATGCAGATCGTTCAAAACAG TCTCG-3′) and MT2R (5′-CCGGAATTCTTAGCATTTGCACTCGCCAGC C-3′) primers introducing BamHI and EcoRI sites (underlined). The PCR products were digested with BamHI and EcoRI and ligated into the yeast expression vector p424 (36) under the transcriptional control of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter. The p424 vector also contains the CYC1 (cytochrome *c* oxidase) terminator, the 2-μ replication origin, and the TRP1 tryptophan marker. Vector p424 and the constructs p424-Hc*MT1* and p424-Hc*MT2* were introduced into *cup1*$^{\Delta}$ and *yap1* cells by using a lithium acetate procedure (46), and transformed cells were selected by their capacity to grow in complete synthetic medium (SD medium), SD medium without Trp (p424 vector selection marker) and Ura (*cup1*$^{\Delta}$ strain selection marker) (SD-Trp-Ura medium), and SD-Trp medium for the *yap1* mutant.

For the functional complementation experiments, cultures of *cup1*$^{\Delta}$ and *yap1* yeast cells carrying either p424 or p424-Hc*MT1* and p424-Hc*MT2* were grown in respective media at 30°C and 220 rpm. Yeast cultures were adjusted to an optical density at 600 nm (OD_{600}) of 1.0, and 5-μl serial dilutions were spotted on plates with SD medium or with SD medium supplemented with 150 μM $CuSO_4$ and 40 μM $CdSO_4$. Plates were incubated for 3 days at 30°C and photographed. In parallel experiments, Falcon jars containing 20 ml of fresh SD-Trp-Ura and SD-Ura media were inoculated with mid-log precultures of *cup1*$^{\Delta}$ and *yap1* cells containing p424, p424-Hc*MT1*, and p424-Hc*MT2* to attain a starting OD_{600} of 0.02. Cells were grown at 30°C and 220 rpm, and $CuSO_4$ (150 μM) and $CdSO_4$ (40 μM) were added 5 h after inoculation. The optical densities of the cultures were measured at 2- to 3-h intervals for 42 h. Furthermore, the transformed yeast cells were tested for their abilities to tolerate high concentrations of Cu and Cd. *cup1*$^{\Delta}$ and *yap1* cells transformed with Hc*MT1* and Hc*MT2* and the empty vector were grown in different concentrations of Cu (200, 400, 600, and 800 μM) and Cd (50, 100, 150, 200, and 250 μM), respectively. Cells grown to log phase were diluted to an OD_{600} of 0.02, and the cell density was measured after 24 h.

Southern blot analysis. To investigate the copy number of Hc*MT1* and Hc*MT2* in the *H. cylindrosporum* genome, Southern blot analysis was performed using genomic DNA. Thirty micrograms of genomic DNA was digested with EcoRV, BamHI, XhoI, or PstI, restriction enzymes which do not cut within the coding sequence of Hc*MT1* or Hc*MT2*. Fragments were size fractioned on 0.8% agarose gels and blotted onto a positively charged nylon membrane using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Membranes were hybridized using the cDNAs of Hc*MT1* and Hc*MT2* as probes. The probe was radiolabeled by random priming using [α-^{32}P]dCTP. Hybridization was performed at 65°C with a solution of 7% (wt/vol) sodium dodecyl sulfate (SDS), 1% bovine serum albumin, 1 mM EDTA, and 0.25 M Na-PO_4 buffer (pH 7.4). Membranes were washed twice in 2× SSC and 0.5% SDS followed by a final wash in 0.2× SSC and 0.1% SDS. The membranes were exposed to a phosphorimage screening and hybridization, and quantification of hybridization signals was done by using a Bio-Rad phosphorimaging system (Bio-Rad, Foster City, CA).

Nucleotide sequence accession numbers. Sequences for Hc*MT1* and Hc*MT2* were deposited in GenBank under accession numbers EU049884 and EU049885, respectively.

RESULTS

Metal tolerance by *H. cylindrosporum*. The growth of *H. cylindrosporum* was adversely affected with increasing concentrations of metals. Increasing the concentration of Cu significantly decreased the growth of the fungus. There was a 50% growth inhibition at 160 μM, and complete growth inhibition occurred at 320 μM. The fungus was very sensitive to Cd; its growth was inhibited by 50% at 21 μM. *H. cylindrosporum* showed a higher tolerance for Zn, and the 50% growth inhibition occurred at 4 mM. The growth of *H. cylindrosporum* decreased as the Pb concentration increased in the medium, and a 50% growth reduction was observed at 50 μM. Ni inhibited the growth of the fungus with increasing concentrations, and a 50% growth reduction was observed at 85 μM for Ni treatment. The growth was completely inhibited at a Ni concentration of 250 μM (Fig. 1).

Identification and sequence analysis of Hc*MT1* and Hc*MT2*. Lambilliotte et al. (28) identified six *H. cylindrosporum* expressed sequence tags (ESTs) corresponding to putative MTs. Sequence analysis showed that they are encoded by two different genes. Corresponding full-length cDNAs were obtained by RACE-PCR and are designated Hc*MT1* and Hc*MT2*. The sequences were deposited in GenBank, as noted above. Both Hc*MT1* and Hc*MT2* contained four exons and three introns. The first exon is very small in both cases (6 to 9 nucleotides long) and all three introns contained conserved intron junctions GT-AG.

The Hc*MT1* cDNA contains a 177-bp open reading frame encoding 59 amino acids (Fig. 2) with a predicted molecular mass of 5.99 kDa and a pI of 4.22. Other characteristics of Hc*MT1*, besides its small size, are the presence of 13 Cys residues (representing 22% of the total amino acid content) with only one aromatic residue (Phe-3). Most of the Cys residues are part of the C-X-C (where X is any amino acid other than Cys) motif, which is supposed to play a role in metal binding. Hc*MT1* contains two C-X-C motifs at the N-terminal end and three at the C-terminal portion, as found in other fungal MTs and also some plant MTs. Hc*MT2* cDNA has an open reading frame of 174 bp encoding a polypeptide containing 57 amino acids with a calculated molecular mass of 5.61 kDa and a pI of 7.95. The Hc*MT2* sequence contains 22.8% of cysteine with no aromatic residues and six C-X-C motifs. Hc*MT1* and Hc*MT2* were 31% identical to each other, and their similarity was 40%. The alignment of Hc*MT1* and Hc*MT2* with other known fungal MTs showed that fungal MTs bear the C-X-C-X(2,3)-C signature at the N-terminal end together with a conserved Cys residue at the C-terminal end. This consensus sequence is far more restricted than the C-G-C-S-X(4)-C-X-C-X(3,4)-C-S-X-C consensus proposed as a signature of fungal MTs by Binz and Kägi (7) (http://www.expasy.ch/cgi-bin/lists?metallo.txt). The alignment presented in Fig. 2 shows that the latter consensus sequence resembles the signature of basidiomycete MTs, which is C-X(3, 4)-C-X-C-X(3)-C-X-C at the N-terminal end together with C-X-C at the C-terminal end.

Induction of Hc*MT1* and Hc*MT2* by different heavy metals. The kinetics of Hc*MT1* and Hc*MT2* transcript accumulation in response to different heavy metals was first studied by transferring the mycelium to a medium supplemented with 320 μM of Cu or 21 μM of Cd, which are potent inducers of MTs. Both genes were induced following a 12-hour treatment with Cu (Fig. 3a). Maximum transcript accumulation was observed at 24 h and decreased thereafter. The highest mRNA accumulation recorded was with Hc*MT1*, which was induced by ca. 350-fold following a 24-hour treatment. Hc*MT2* was slightly less sensitive to Cu induction; at 24 h, the transcript level was ca. 100 times higher than that of the control. Hc*MT2* was also

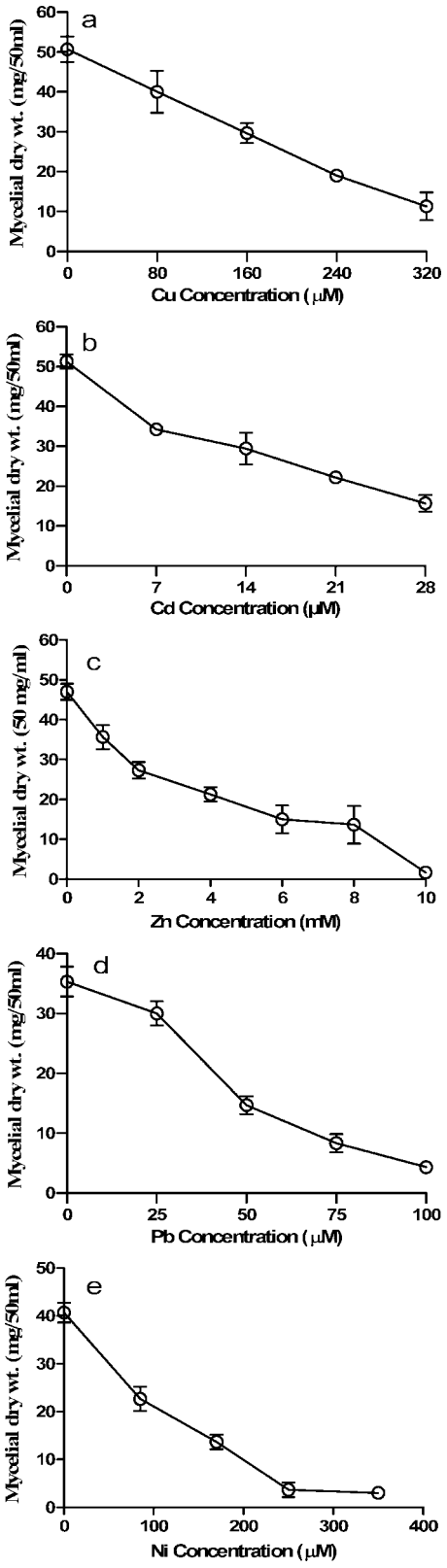

FIG. 1. Effect of different concentrations of Cu (a), Cd (b), Zn (c), Pb (d), and Ni (e) on the mycelial growth of *H. cylindrosporum*. The metals were amended after 3 days of fungal inoculation to avoid the immediate stress and also to allow the fungus to initiate the growth. Shown are means ± standard errors of the means (error bars).

induced by Cd. Transcript accumulation was at its maximum at 48 h of treatment (Fig. 3b). It was ca. 40 times as high as that of the control (zero time). In contrast, Hc*MT1* was not induced by Cd. To investigate the response of MT genes to oxidative stress, the fungal culture was grown in liquid medium supplemented with H_2O_2. Expression levels of both Hc*MT1* and Hc*MT2* were increased in the presence of oxidative stress. The maximum induction was observed at 48 h and at 36 h for Hc*MT1* and Hc*MT2*, respectively (Fig. 3c). However, the induction levels were not high compared to the expression levels under Cu stress, suggesting that the expression of Hc*MT1* and Hc*MT2* is more specific in response to metals than oxidative stress.

In an attempt to further document the regulation of Hc*MT1* and Hc*MT2*, the effects of various Cu or Cd concentrations on the expression levels of Hc*MT1* and Hc*MT2* were studied after a 24-h treatment. The induction of Hc*MT1* increased as a function of Cu concentration. It was more than 300-fold above control levels when the Cu concentration was higher than 240 μM. Irrespective of Cu concentrations, Hc*MT2* was much less sensitive to Cu induction than Hc*MT1*. The mRNA accumulation of Hc*MT2* was increased as the concentration of Cu increased, and the maximum accumulation was recorded at 320 μM, where it was almost 100 times higher than that of the control (Fig. 4a). Cadmium-induced transcription of Hc*MT2* increased as a function of metal concentration up to a maximum of 21 μM (Fig. 4b). No induction could be detected in the presence of 28 μM of Cd, which was highly inhibitory to fungal growth. Hc*MT1* expression was not induced by any of the concentrations of Cd used (Fig. 4b), which confirms the absence of induction shown in Fig. 3b. Zinc is a potent inducer of MTs in higher eukaryotes. For this reason, we tested the effect of a wide range of Zn concentrations on *HcMT* gene expression by using Zn along with other heavy metals, such as Pb and Ni. At different concentrations of Zn, Pb, and Ni, none of these metals induced the expression of Hc*MT1* or Hc*MT2* genes, although fungal growth was inhibited by the concentrations shown in Fig. 1c to e.

To validate the functional roles of Hc*MT1* and Hc*MT2*, these genes were expressed in *S. cerevisiae* mutant strains which are unable to grow with high concentrations of various metals, and growth was then monitored for both the control and the metal-supplemented media. The *S. cerevisiae yap1* locus encodes a transcription factor related to the mammalian AP-1 complex that positively controls various genes involved in metal tolerance and, more generally, oxidative stress tolerance in yeast (25). Although yeast MT genes are not direct targets of such an activator, *yap1* mutants are particularly sensitive to Cd and are, thus, suitable to highlight tolerant phenotypes induced by exogenous cDNAs (52). In *S. cerevisiae*, *CUP1* encodes a Cu-thionein that is induced by and binds to Cu. Most laboratory yeast strains contain multiple copies of this gene, but the *cup1*^{s} strain harbors a single copy of the MT gene, and its growth is inhibited at a 300 μM concentration of $CuSO_4$. The Cu tolerance threshold is reduced to 75 μM $CuSO_4$ in the *cup1*$^\Delta$ strain (*cup1*::*URA3*), which has no functional copies of *CUP1* (18). As shown in Fig. 5a, 150 μM $CuSO_4$ inhibited the growth of p424 *cup1*$^\Delta$ cells, whereas the same cells carrying p424-Hc*MT1* and p424-Hc*MT2* were able to grow at a rate similar to that of parental *cup1*^{s} cells. Figure 6a shows that the *yap1* strain trans-

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P. tinctorius                MQSVNAVLVNNNDKCGSAACTCGSSCACKPG----------------------------------ECKC
P. involutus                  MNTITSVPVNFNNCGSNSCGCGSSCACKPG----------------------------------ECKC
L. edodes                     MSTTTQTPVSQANCGSSSCSCGDSCACQPN----------------------------------ECKC
H. cylindrosporum HcMT.1        MQFTSILVNQACGSDNCQCDAACTCSSGSCHAPVNR--------------ACGSSDCNCNSSCCGCESNNCNCN
H. cylindrosporum HcMT.2      MQIVQNSLVSQSSGCTCTSCKCGSNCTCGAPVNQSS-------------GCGSSSCTCTS TCKAGECKC
A. bisporus                       MPATTCASKCG-EACACANNCQCCSNNE---VPKNQ-------HCGMSS GCGDSCGCKPDECKC
A. bisporus                       MPATMCTFKCG-EACACTNNCQCRKDNNNSTVPENHG------HCGIDG NCGDACACTREGCKCH
P. anserina                        MGGSCNCSGSASSCSCGSDCS-----------------------------------------CSGCK
N. crassa                            MGDCGCSGASSCNCGSGCSCSN----------------------------------------CGSK
S. cerevisiae Crs5               MTVKICDCGECCKDSCHCGSTCLPSCSGGEKCKCDHSTGS--------PQCKSCGEKCKCETTCTCEKSKCNCEKC
S. cerevisiae CUP1               MFSELINFQNEGHECQCQCGS-CKNNEQCQKSCSCPTG-------CNSDDKCPCGNKSEETKKSCCSGK
G. margarita MT-1                   MSSGCSCGS-GCKCGDNCSCSMYPDMETNTTVTMIEGVAPLKMYSEGSEKSFGAEGGNGCKCGSNCKCDPCNC
G. margarita GmarMT1                MCQNCKCGS-ACQCGTNCTCPKDYTTSSTTQQQQQSTDT---QKKTASCGVSTCRCGEVCKCTKGNCKC
```

FIG. 2. Multiple alignment of fungal metallothionein protein sequences. Gaps introduced to provide the best alignment are indicated by dashes. Fully conserved Cys residues are boxed with a black background; Cys residues conserved in basidiomycetes are boxed with a gray background. The NCBI accession numbers are as follows: *Pisolithus tinctorius*, CB010976; *P. involutus*, AAS19463; *Lentinula edodes*, CO501612, *H. cylindrosporum* Hc*MT1* strain, EU049884; *H. cylindrosporum* Hc*MT2* strain, EU049885; *A. bisporus*, CAC85298; *A. bisporus*, CAC85299; *P. anserina*, CAA06385; *N. crassa*, P02807; *S. cerevisiae* Crs5, YOR031W; *S. cerevisiae* CUP1, YHR055C; *G. margarita* MT1, CAD13456; *G. margarita* GmarMT1, AJ421527.

formed with the empty vector was unable to grow with 40 μM Cd, whereas the Cd-sensitive phenotype of the *yap1* mutants was complemented by Hc*MT1* and Hc*MT2* (Fig. 6a). Further, the restoration of Cd and Cu tolerance by the expression of Hc*MT1* and Hc*MT2* was confirmed with liquid culture assays.

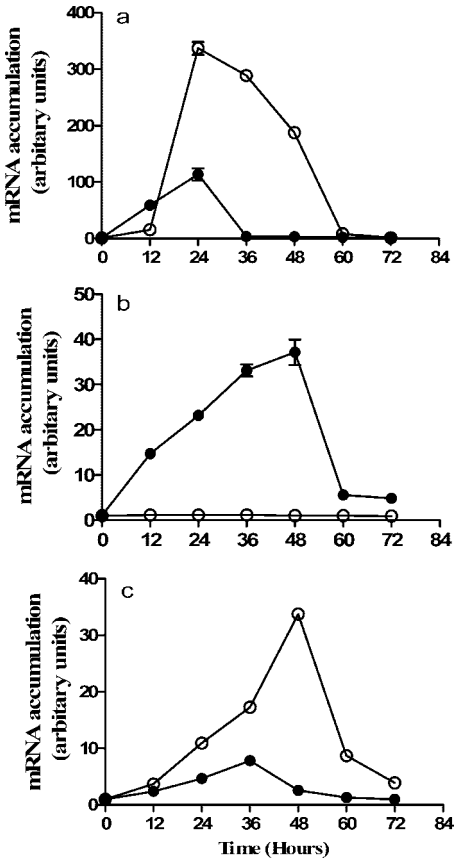

FIG. 3. Expression of Hc*MT1* (open circles) and Hc*MT2* (filled circles) in *H. cylindrosporum* after incubation in medium supplemented with 320 μM of Cu (a), 21 μM of Cd (b), and 25 mM of H_2O_2 (c) for different time intervals. Transcript accumulation was quantified by cPCR and expressed as arbitrary units, where 1 is transcript level at zero time. Bars indicate standard errors ($P \leq 0.05$).

As shown in Fig. 5 and 6, p424-Hc*MT1* and *HcMT2 cup1*$^{\Delta}$ and *yap1* cells grew well in medium containing 150 μM $CuSO_4$ and 40 μM $CdSO_4$, whereas the growth of empty vector-transformed cells was inhibited. However, p424 *cup1*$^{\Delta}$ and p424 *yap1* showed normal growth when cultured in a Cu- and Cd-free medium (Fig. 5b and 6b). The Hc*MT1*- and Hc*MT2*-transformed cells of *cup1*$^{\Delta}$ were able to grow in the presence of Cu concentrations of up to 400 μM. Concentrations higher than 500 μM fully inhibited the growth of yeast cells. The cells transformed with the empty vector did not show any significant growth in any of the concentrations tested (Fig. 7a). The *yap1* cells transformed with Hc*MT1* did not show any increased tolerance to Cd compared to cells transformed with the empty vector (Fig. 7b). In contrast, Hc*MT2*-transformed cells signif-

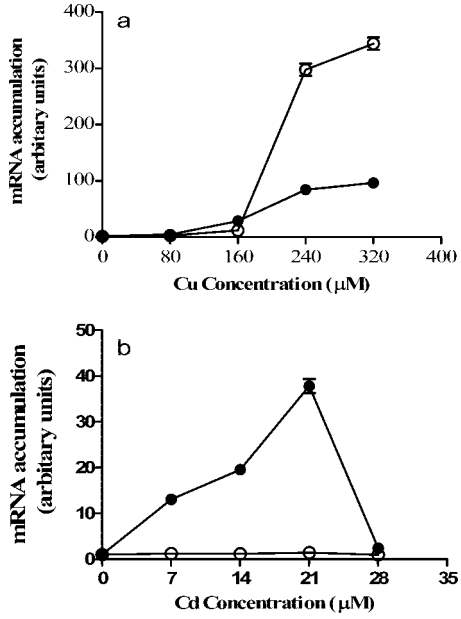

FIG. 4. Expression of Hc*MT1* (open circles) and Hc*MT2* (filled circles) in *H. cylindrosporum* after 24 h of incubation in medium supplemented with different concentrations of Cu (a) and Cd (b). Transcript accumulation was quantified and expressed as described in the legend to Fig. 3. Bars indicate standard errors ($P \leq 0.05$).

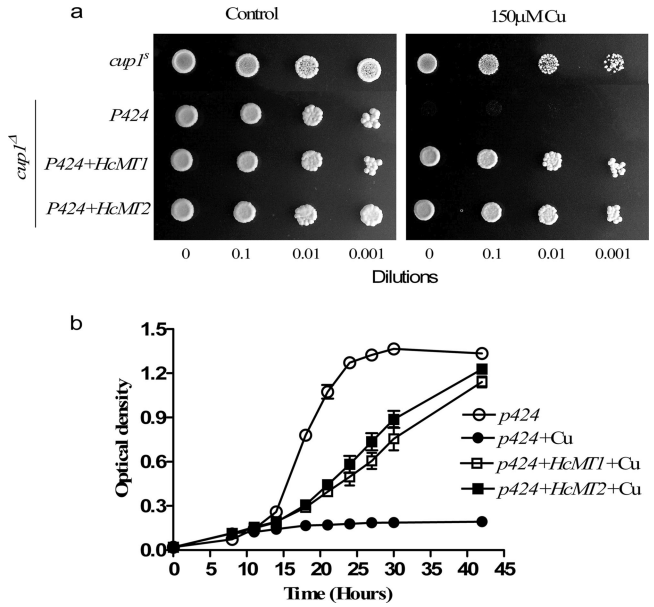

FIG. 5. Functional complementation and growth of the yeast mutants on selective media. (a) Cu tolerance of *cup1*$^{\Delta}$ cells expressing Hc*MT1* and Hc*MT2*. *CUP1*-null cells harboring the control vector p424 (*cup1*$^{\Delta}$) or the constructs p424-Hc*MT1* and p424-Hc*MT2* and parental cells with a single CUP1 gene copy (*cup1*^{s}) were spotted on SD medium supplemented (or not) with 150 μM Cu. (b) Growth curve of *cup1*$^{\Delta}$ cells in SD-Trp medium transformed with the empty vector p424 or with p424-Hc*MT1* and p424-Hc*MT2*. The *cup1*^{s} strain harbors a single copy of this MT gene, and its growth is inhibited at 300 μM $CuSO_4$. The Cu tolerance threshold is reduced to 75 μM $CuSO_4$ in the *cup1*$^{\Delta}$ strain supplemented (or not) with 150 μM Cu.

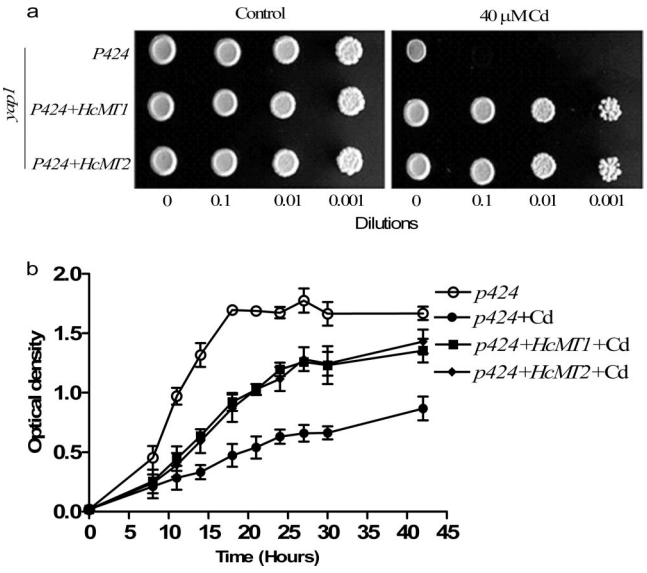

FIG. 6. Functional complementation and growth of the yeast mutants on selective media. (a) *yap1* mutant cells were transformed with the empty vector p424 or with p424-Hc*MT1* and p424-Hc*MT2*. Yeast cells were spotted on SD medium supplemented (or not) with 40 μM Cd. (b) Growth curve of *yap1* cells in SD-Trp medium transformed with the empty vector p424 or with p424-Hc*MT1* and p424-Hc*MT2* supplemented (or not) with 40 μM Cd.

icantly improved the growth of yeast cells in the presence of Cd concentrations of up to 100 μM. These results suggest that the two genes provide equivalent tolerances to Cu, whereas Hc*MT2* alone provides tolerance to Cd.

Promoter analysis of Hc*MT1* and Hc*MT2*. In order to explain whether the differential expression of Hc*MT1* and Hc*MT2* might be due to differential regulation, we performed computational analyses of the respective upstream regions of their promoters. Both Hc*MT1* and Hc*MT2* promoters contained the standard stress response elements implicated in metal response, such as metal response element (designated MRE) and general stress response (GATA), response to phosphate starvation (PHO), response to nitrogen utilization (NIT), and heat shock (HSF) elements. However, the numbers and varieties of potential regulatory elements in Hc*MT1* and Hc*MT2* promoter regions were different. The Hc*MT1* promoter contained response elements that were not present in the Hc*MT2* promoter region; these included STRE, which is known to be responsible for the multiple stress response or to enhance the response of other elements, and GCN, which responds to amino acid- and nitrogen starvation-related stress. On the other hand, the Hc*MT2* promoter contained several additional regulatory elements, such as GCR, which is involved in regulating glycolytic enzymes, presumably in response to heavy metal stress in the production of organic acids; DRE, which is involved in drug or heavy metal efflux mechanisms; and MCM, which may be involved in osmosensing and in heavy metal tolerance-induced pathways. The differences in and locations of potential regulatory elements in Hc*MT1* and Hc*MT2* promoter regions might be the reasons for the different regulation patterns.

Organization of Hc*MT1* and Hc*MT2* in the fungal genome. The copy numbers of Hc*MT1* and Hc*MT2* genes in *H. cylindrosporum* were analyzed by Southern blot analysis. Hybridiza-

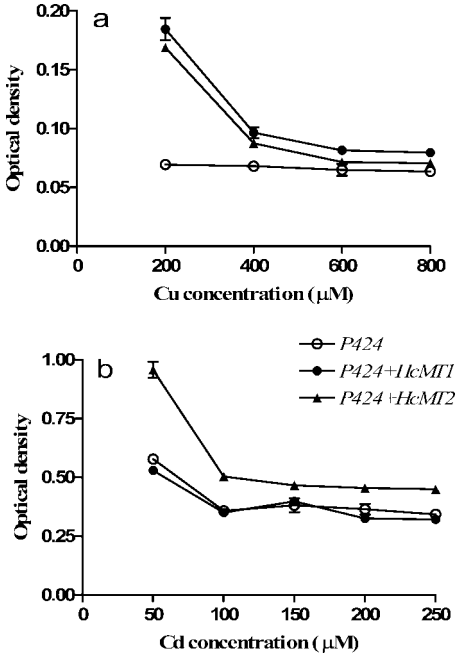

FIG. 7. Increased metal tolerance conferred by Hc*MT1* and Hc*MT2* in metal-hypersensitive yeast mutants. *cup1*$^{\Delta}$ (a) and *yap1* (b) yeast mutants harboring p424-Hc*MT1*, p424-Hc*MT2*, and an empty vector (p424) were grown in SD medium with different concentrations of Cu and Cd.

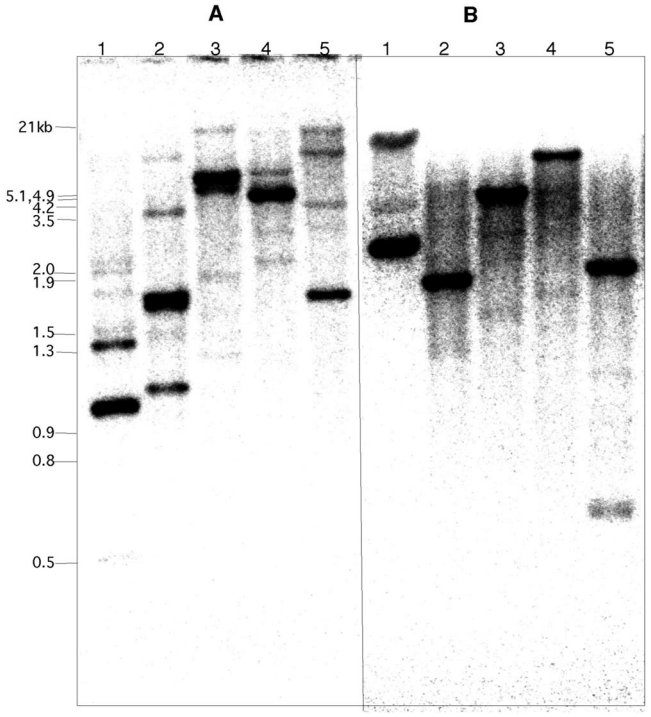

FIG. 8. Southern blot analysis of gene copy numbers for Hc*MT1* (A) and Hc*MT2* (B). Genomic DNA was digested with the indicated restriction enzymes, and DNA fragments were separated on a 0.8% agarose gel, transferred to nylon membranes, and hybridized with a radiolabeled Hc*MT1* or Hc*MT2* probe. The membranes were washed at high stringency and exposed to phosphorimaging screens and scanned using a Bio-Rad personal phosphorimager scanner. Lanes 1, marker (λ DNA digested with HindIII), lanes 2, EcoRI; lanes 3, BamHI; lanes 4, XhoI; lanes 5, PstI.

tion experiments using Hc*MT1* and Hc*MT2* insertion fragments as probes revealed single hybridization fragments when the genomic DNA was digested with different enzymes (Fig. 8). These results showed that both genes exist as a single copy in the genome of *H. cylindrosporum*.

DISCUSSION

The aim of this work was to characterize different MTs of the ectomycorrhizal fungus *H. cylindrosporum* and to study the specificities of their induction in an attempt to determine their possible functional roles in heavy metal detoxification. We identified two different MTs which show similarity to fungal MTs. This is the first report of the presence of a multigene family encoding MTs in an ectomycorrhizal fungus. As MTs are generally not identified in genome sequencing projects, the present results show that EST methods can be a suitable alternative to identify MTs. Out of ca. 4,500 ESTs (corresponding to ca. 3,500 unigenes) isolated by Lambilliotte et al. (28) from *H. cylindrosporum* grown in the absence of heavy metals and stress, we identified six MT ESTs corresponding to the two identified genes. The fact that 77% of the ESTs in the library exist as less than three copies indicates that Hc*MT1* and Hc*MT2* are expressed at a high basal level. In contrast, transcripts of Pi*MT1* were almost undetectable in *P. involutus* grown in the absence of heavy metal (6).

Differential regulation of Hc*MT1* and Hc*MT2*. Higher-eukaryote MTs are generally induced by a wide array of metals and stress conditions (10). In contrast, each of the fungal MTs studied so far is induced by a limited number of heavy metals, with most of them being induced by Cu (11, 14, 27, 30, 37). This was also the case for both MTs characterized in this work. However, Hc*MT1* was specifically regulated by Cu, not by Cd, Zn, Pb, and Ni, whereas Hc*MT2* showed a dual regulation, since it was induced by Cu and Cd. The *Neurospora crassa* MT characterized by Cobine et al. (11) and Münger et al. (39) was inducible by Cu, not by Cd, Zn, Co, or Ni. Likewise, the *P. anserina* MT was upregulated by Cu, not by Cd, Zn, or Mn (2). In *C. glabrata*, both the genes coding for MTs were induced by Cu and Ag but not by Cd (32); the Hc*MT1* of *H. cylindrosporum* can also be classified in this group. This is not the case for Hc*MT2*, which is also induced by Cd.

Cadmium-inducible MTs have been identified for different fungi (6, 13, 20, 21, 22, 29). Lanfranco et al. (29) and Bellion et al. (6) showed that the Cd-inducible MTs of *Beauveria bassiana*, *G. margarita*, and *P. involutus* are also induced by Cu. This is also the case for Hc*MT2*, the regulation of which appears to be similar to that of Pi*MT1* from *P. involutus* (6), both being induced by Cu and Cd but not by Zn. As mentioned by these authors, in the case of Pi*MT1*, the encoded proteins could be involved in the response of mycorrhizal fungi to Cu and Cd stress. This hypothesis can be updated on the basis of the present results. Indeed, Hc*MT1* is much more responsive to Cu induction than is Hc*MT2*, and Hc*MT1* is not induced by Cd. This suggests that Hc*MT1* could be specifically involved in Cu detoxification, whereas Hc*MT2*, like Pi*MT1*, which shows a dual regulation, could have a more pleiotropic role. The time frame for transcript accumulation was studied for both Hc*MT1* and Hc*MT2* genes in the presence of Cu and Cd. Both genes were induced following 12 h of treatment with Cu, and maximum transcript accumulation was observed at 24 h. Hc*MT2* transcript accumulation was induced following 12 h treatment with Cd, and maximum accumulation was attained at 48 h, though the level of accumulation was significantly lower than that seen with Cu treatment. Hc*MT1* did not respond to Cd in all the time intervals tested. These results suggest that both genes seem to respond faster and more strongly to Cu than to Cd, because Cd detoxification may also involve other mechanisms, such as phytochelatins. The growth rate of the fungus was also altered depending on the concentrations of Cu and Cd used during the treatments. Based on these results, we can hypothesize that this growth inhibition could account for the response to the metal supply.

Although some MTs of higher eukaryotes have been shown to bind Zn (10), there are only few reports of Zn-inducible MTs in fungi. Pagani et al. (41), however, reported the involvement of the *Crs5* Cu-metallothionein of *S. cerevisiae* in Zn detoxification. Tucker et al. (47) described an MT-like protein in *Magnaporthe grisea* which showed a very high affinity to Zn. Based on these reports, although none of the MTs identified to date in ectomycorrhizal fungi is induced by Zn, the presence of a Zn-specific MT(s) in these fungi (including *H. cylindrosporum*) cannot be ruled out.

Agents capable of mediating the formation of free radicals are known to induce MT mRNAs (4). For example, a plant type 2 metallothionein was reported by Mir et al. to respond to

oxidative stress (35). González-Guerrero et al. (17) reported the role of Gint*MT1* in the regulation of the redox status of the extraradical mycelium of *Glomus intraradices* through either its metal chelation activity or its thiol groups. In this study, also Hc*MT1* and, to a lesser extent, Hc*MT2* were induced in response to oxidative stress. These results suggest a role for *H. cylindrosporum* MTs in maintaining the local redox balance, either by sequestering copper or by otherwise preventing potentially deleterious Fenton chemistry reactions (54).

Functional roles of Hc*MT1* and Hc*MT2*. To demonstrate the roles of Hc*MT1* and Hc*MT2* in metal detoxification, yeast complementation assays were performed for this study. Heterologous complementation assays of yeast revealed that Hc*MT1* and Hc*MT2* encode functional polypeptides capable of conferring increased tolerance against Cu and Cd. Growth studies of yeast strains complemented with Hc*MT1* or Hc*MT2* suggest that both genes are able to provide tolerance to Cu, whereas only Hc*MT2* is involved in conferring tolerance to Cd. These results could be correlated with the expression studies of *H. cylindrosporum* in the presence of different concentrations of Cu and Cd tested, in which both genes were expressed in the presence of Cu and only Hc*MT2* responded to Cd.

In conclusion, our results show experimental proof that ectomycorrhizal fungi, such as *H. cylindrosporum*, encode different MTs, with differences in patterns of regulation. Hc*MT1* is highly responsive to Cu induction and is likely to be involved in the detoxification of this metal. There are indeed differences between the promoter sequences of Hc*MT1* and Hc*MT2*, and these may be the basis for differences in transcript levels. Based on sequence analyses, and on the criteria suggested for promoter evolution (34, 42, 53), it appears that Hc*MT2* likely originates from duplication of Hc*MT1* followed by changes in its sequence to acquire new elements, such as DRE elements, as well as duplication of existing elements, such as PHO. It has been shown that cells respond to multiple stresses in a synergistic manner, and exposure to one form of stress leads to transient stress hardening or cross-tolerance to other forms of stress (26). Thus, duplication and acquisition of new stress response elements facilitate additional adaptations that are stressor specific and aimed at reestablishing cellular homeostasis. The additional stress response elements in addition to metal response elements in both Hc*MT1* and Hc*MT2* suggest differential regulation characteristics and functions for Hc*MT1* and Hc*MT2*. Thus, Hc*MT2* exhibits a more pleiotropic role, similar to that of Pi*MT1* of *P. involutus*. The differences in the elements occurring in promoters of Hc*MT1* and Hc*MT2* suggest diversification of stress response functions and response levels between Hc*MT1* and Hc*MT2*, as seen from the expression analyses.

Altogether, these results show that the identification and functional verification of different MTs in ectomycorrhizal fungi and the study of their regulation is a prerequisite for a better understanding of heavy metal tolerance of these fungi and, subsequently, for the determination of their abilities to detoxify and improve heavy metal tolerance of their host plants.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Averbeck, N. B., C. Borghouts, A. Hamann, V. Specke, and H. D. Osiewacz.** 2001. Molecular control of copper homeostasis in filamentous fungi: increased expression of a metallothionein gene during aging of *Podospora anserina*. Mol. Gen. Genet. **264:**604–612.
3. **Ayres, R. U.** 1992. Toxic heavy metals: materials cycle optimization. Proc. Natl. Acad. Sci. USA **89:**815–820.
4. **Bauman, J. W., J. Liu, and C. D. Klaassen.** 1991. Increase in metallothionein produced by chemicals that induce oxidative stress. Toxicol. Appl. Pharmacol. **110:**347–354.
5. **Bellion, M., M. Courbot, C. Jacob, D. Blaudez, and M. Chalot.** 2006. Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiol. Lett. **254:**173–181.
6. **Bellion, M., M. Courbot, C. Jacob, F. Guinet, D. Blaudez, and M. Chalot.** 2007. Metal induction of a *Paxillus involutus* metallothionein and its heterologous expression in *Hebeloma cylindrosporum*. New Phytol. **174:**151–158.
7. **Binz, P. A., and J. H. R. Kägi.** 1999. Metallothionein: molecular evolution and classification. Birkhäuser-Verlag, Basel, Switzerland.
8. **Butt, T. R., E. J. Sternberg, J. A. Gorman, J. A. Clark, D. Hamer, M. Rosenberg, and S. T. Crooke.** 1984. Copper metallothionein of yeast, structure of the gene, and regulation of expression. Proc. Natl. Acad. Sci. USA **81:**3332–3336.
9. **Cartharius, K., K. Frech, K. Grote, B. Klocke, M. Haltmeier, A. Klingenhoff, M. Frisch, M. Bayerlein, and T. Werner.** 2005. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics **21:**2933–2942.
10. **Cobbett, C., and P. Goldsbrough.** 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu. Rev. Plant Biol. **53:**159–182.
11. **Cobine, P. A., R. T. McKay, K. Zangger, C. T. Dameron, and I. M. Armitage.** 2004. Solution structure of Cu6 metallothionein from the fungus *Neurospora crassa*. Eur. J. Biochem. **271:**4213–4221.
12. **Colpaert, J. V., and J. A. van Assche.** 1993. The effects of cadmium on ectomycorrhizal *Pinus sylvestris* L. New Phytol. **123:**325–333.
13. **Courbot, M., M. Chalot, L. Diez, P. Leroy, and R. Ruotolo.** 2004. Cadmium responsive thiols in the ectomycorrhizal fungus *Paxillus involutus*. Appl. Environ. Microbiol. **70:**7413–7417.
14. **Culotta, V. C., W. R. Howard, and X. F. Liu.** 1994. *CRS5* encodes a metallothionein-like protein in *Saccharomyces cerevisiae*. J. Biol. Chem. **269:**25295–25302.
15. **Dixon, R. K., and C. A. Buschena.** 1988. Response of ectomycorrhizal *Pinus banksiana* and *Picea glauca* to heavy metals in soil. Plant Soil **105:**265–271.
16. **Gadd, G. M.** 1993. Interactions of fungi with toxic metals. New Phytol. **124:**25–60.
17. **González-Guerrero, M., C. Cano, C. Azcón-Aguilar, and N. Ferrol.** 2007. *GintMT1* encodes a functional metallothionein in *Glomus intraradices* that responds to oxidative stress. Mycorrhiza **17:**327–335.
18. **Hamer, D. H., D. J. Thiele, and J. E. Lemontt.** 1985. Function and autoregulation of yeast copper thionein. Science **228:**685–690.
19. **Howe, R., R. L. Evans, and S. W. Ketteridge.** 1997. Copper-binding proteins in ectomycorrhizal fungi. New Phytol. **135:**123–131.
20. **Hwang, C. S., and P. E. Kolattukudy.** 1995. Isolation and characterization of genes expressed uniquely during appressorium formation by *Colletotrichum gloeosporioides* conidia induced by the host surface wax. Mol. Gen. Genet. **247:**282–294.
21. **Jacob, C., M. Courbot, F. Martin, A. Brun, and M. Chalot.** 2004. Transcriptomic response to cadmium in the ectomycorrhizal fungus *Paxillus involutus*. FEBS Lett. **576:**423–427.
22. **Jaeckel, P., G. Krauss, S. Menge, A. Schierhorn, P. Rucknagel, and G. J. Krauss.** 2005. Cadmium induces a novel metallothionein and phytochelatin 2 in an aquatic fungus. Biochem. Biophys. Res. Commun. **333:**150–155.
23. **Jargeat, P., D. Rekangalt, M. C. Verner, G. Gay, J. C. Debaud, R. Marmeisse, and L. Fraissinet-Tachet.** 2003. Characterisation and expression analysis of a nitrate transporter and nitrite reductase genes, two members of a gene cluster for nitrate assimilation from the symbiotic basidiomycete *Hebeloma cylindrosporum*. Curr. Genet. **43:**199–205.
24. **Karin, M., and R. I. Richards.** 1984. The human metallothionein gene family: structure and expression. Environ. Health Perspect. **54:**111–115.
25. **Kuge, S., and N. Jones.** 1994. YAP1 dependent activation of TRX2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. EMBO J. **13:**655–664.
26. **Kültz, D.** 2003. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. J. Exp. Biol. **206:**3119–3124.
27. **Kumar, K. S., S. Dayananda, and C. Subramanyam.** 2005. Copper alone, but not oxidative stress, induces copper-metallothionein gene in *Neurospora crassa*. FEMS Microbiol. Lett. **42:**45–50.

28. **Lambilliotte, R., R. Cooke, D. Samson, C. Fizames, F. Gaymard, C. Plassard, M.-V. Tatry, C. Berger, M. Laudié, F. Legeai, E. Karsenty, M. Delseny, S. Zimmermann, and H. Sentenac.** 2004. Large-scale identification of genes in the fungus *Hebeloma cylindrosporum* paves the way to molecular analyses of ectomycorrhizal symbiosis. New Phytol. **164:**505–513.
29. **Lanfranco, L., A. Bolchi, E. C. Ros, S. Ottonello, and P. Bonfante.** 2002. Differential expression of a metallothionein gene during the presymbiotic versus the symbiotic phase of an arbuscular mycorrhizal fungus. Plant Physiol. **130:**58–67.
30. **Lerch, K.** 1980. Copper metallothionein, a copper-binding protein from *Neurospora crassa*. Nature **284:**368–370.
31. **Longo, V. D., E. B. Gralla, and J. S. Valentine.** 1996. Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. J. Biol. Chem. **271:**12275–12280.
32. **Mehra, R. K., J. R. Garey, T. R. Butt, W. R. Gray, and D. R. Winge.** 1989. *Candida glabrata* metallothioneins: cloning and sequence of the genes and characterization of proteins. J. Biol. Chem. **264:**19747–19753.
33. **Melin, E.** 1953. Physiology of mycorrhizal relations in plants. Annu. Rev. Plant Physiol. **4:**325–346.
34. **Miller, C., J. McDonald, and D. Francis.** 1996. Evolution of promoter sequences: elements of a canonical promoter for prespore genes of *Dictyostelium*. J. Mol. Evol. **3:**185–193.
35. **Mir, G., J. Domènch, G. Hugue, W. J. Guo, and P. Goldsbrough.** 2004. A plant type 2 metallothionein (MT) from cork tissue responds to oxidative stress. J. Exp. Bot. **55:**2483–2493.
36. **Mumberg, D., R. Muller, and M. Funk.** 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene **156:**119–122.
37. **Münger, K., and K. Lerch.** 1985. Copper metallothionein from the fungus *Agaricus bisporus*: chemical and spectroscopic properties. Biochemistry **24:** 6751–6756.
38. **Münger, K., U. A. Germann, and K. Lerch.** 1985. Isolation and structural organization of the *Neurospora crassa* copper metallothionein gene. EMBO J. **4:**2665–2668.
39. **Münger, K., U. A. Germann, and K. Lerch.** 1987. The *Neurospora crassa* metallothionein gene: regulation of expression and chromosomal location. J. Biol. Chem. **262:**7363–7367.
40. **Okuyama, M., Y. Kobayashi, M. Inouhe, H. Tohoyama, and M. Joho.** 1999. Effect of some heavy metal ions on copper-induced metallothionein synthesis in the yeast *Saccharomyces cerevisiae*. Biometals **12:**307–314.
41. **Pagani, A., L. Villarreal, M. Capdevila, and S. Atrain.** 2007. The *Saccharomyces cerevisiae* Crs5 metallothionein metal binding abilities and its role in the response to zinc overload. Mol. Microbiol. **63:**256–269.
42. **Raijman, D., R. Shamir, and A. Tanay.** 2008. Evolution and selection in yeast promoters: analyzing the combined effect of diverse transcription factor binding sites. PLoS Comput. Biol. **4:**77–87.
43. **Rouillon, R., G. Gay, J. Bernillon, J. Favre-Bonvin, and G. Bruchet.** 1986. Analysis by HPLC-mass spectrometry of the indole compounds released by the ectomycorrhizal fungus *Hebeloma hiemale* in pure culture. Can. J. Bot. **64:**1893–1897.
44. **Singh, G., and A. M. Ashby.** 1998. Cloning of the mating type loci from *Pyrenopeziza brassicae* reveals the presence of a novel mating type gene within a discomycete MAT 1–2 locus encoding a putative metallothionein-like protein. Mol. Microbiol. **30:**799–806.
45. **Smith, S., and D. J. Read.** 1997. Mycorrhizal symbiosis. Academic Press, San Diego, CA.
46. **Stearns, T., H. Ma, and D. Botstein.** 1990. Manipulating yeast genome using plasmid vectors. Methods Enzymol. **185:**280–297.
47. **Tucker, S. L., C. R. Thornton, K. Tasker, C. Jacob, G. Giles, M. Egan, and N. J. Talbot.** 2004. A fungal metallothionein is required for pathogenicity of *Magnaporthe grisae*. Plant Cell **16:**1575–1588.
48. **van Kan, J. A. L., G. F. J. M. van den Ackerveken, and P. J. G. M. de Wit.** 1991. Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal pathogen *Cladosporium fulvum*, casual agent of tomato leaf mold. Mol. Plant-Microbe Interact. **4:**52–59.
49. **Waalkes, M. P., and P. L. Goering.** 1990. Metallothionein and other cadmium-binding proteins: recent developments. Chem. Res. Toxicol. **3:**281–288.
50. **Wagner, F., G. Gay, and J. C. Debaud.** 1988. Genetic variation of nitrate reductase activity in mono- and dikaryotic populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum* Romagnési. New Phytol. **113:**259–264.
51. **Wingender, E., X. Chen, E. Fricke, R. Geffers, R. Hehl, I. Liebich, M. Krull, V. Matys, H. Michael, R. Ohnhäuser, M. Prüss, F. Schacherer, S. Thiele, and S. Urbach.** 2001. The TRANSFAC system on gene expression regulation. Nucleic Acids Res. **29:**281–283.
52. **Wu, A., J. A. Wemmie, N. P. Edginton, M. Goeb, J. L. Guevara, and W. S. Moye-Rowley.** 1993. Yeast bZip proteins mediate pleiotropic drug and metal resistance. J. Biol. Chem. **268:**18850–18858.
53. **Zhang, J.** 2003. Evolution by gene duplication: an update. Trends Ecol. Evol. **18:**292–298.
54. **Zhang, S., J. Li, C. C. Wang, and C. L. Tsou.** 1999. Metal regulation of metallothionein participation in redox reactions. FEBS Lett. **462:**383–386.